Chapter 8

General Discussion
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Combinations of osteogenic agents for bone regeneration in adverse conditions

1. The efficacy of different BMPs on osteoblastogenesis in alcoholism

The osteo-restoration of large bone defects remains a challenge, particularly for patients with adverse systemic conditions, such as alcoholism. Highly concentrated ATRA is regarded as one of the important mediators of alcoholism-related osteoporosis [1] and compromised osteogenesis [2]. In our previous studies, we showed that heterodimeric BMP2/7, a more potent form than homodimeric BMPs, could antagonize the inhibitory effect of ATRA and rescue in vitro osteoblastogenesis of both immortalized preosteoblast cells [3] and primary bone marrow mesenchymal stem cells [4]. However, it is not yet clear whether such an effect of BMP2/7 is also advantageous over those of BMP2 and BMP7. In chapter 2, we first compared the efficacy of heterodimeric BMP2/7 and homodimeric BMPs in antagonizing the inhibitory effect of ATRA on in vitro osteoblastogenesis. Interestingly, our data showed that the antagonism by BMP2/7 of ATRA was not superior to that of homodimeric BMP2 or BMP7.

ATRA-inhibited osteoblastogenesis was previously characterized as a partially differentiated non-proliferating state [5]. These characteristics were based on the findings from our and other researchers’ studies in which ATRA could suppress cell proliferation and viability, OCN expression, and mineralized nodules of osteoblasts, but either had no influence on, or even promoted, the ALP activity of MC3T3-E1 pre-osteoblasts [3, 5] and primary mouse BMSCs [4, 6]. Consistently, RA was also shown to promote ALP activity in C3H10T1/2 MSCs [7, 8] and the rat preosteoblast cell line UMR-201-10B [9], but suppressed the mRNA expression of Osterix and osteocalcin in C3H10T1/2 cells [8]. By contrast, ATRA suppressed the adipogenic differentiation and promoted the osteogenic differentiation of adipose-derived stromal cells or partially differentiated preadipocytes [10, 11]. Such an effect of ATRA was highly dependent on a RARE (retinoid acid response element) in the promoter of the ALP gene [8]. Besides, the RA-induced osteogenic differentiation of ADAS seemed to depend on RA-induced TGF-β signaling: RA enhanced the endogenous expression of TGF-β1 and, especially, TGF-β2 [7]. Furthermore, ATRA enhanced the endogenous expression of SMAD3, a key TGF-β signaling transcription factor, and its translocation in nuclei, thereby initiating the displacement of a negative regulatory transcription factor, CCAAT/Enhancer Binding Protein beta (C/EBPβ), from the RUNX2 promoter [12]. Contrastingly, TGF-β was shown to inhibit BMP-2 induced osteoblastogenesis [13], which might be the key mechanism accounting for the complicated and ambiguous effect of ATRA on osteoblastogenesis in different cell types.

In chapter 2, we showed that BMP2/7, but not BMP2 or BMP7, could restore the cell viability that was suppressed by ATRA. Our results showed that the effect of ATRA did not take place immediately after treatment. One potential mechanism is that ATRA binds to and inhibits adenine nucleotide translocase and induces mitochondrial permeability transition via as-yet-unknown mechanisms [14]. Our previous studies showed that 50 ng/ml of BMP2/7 could enhance the osteoblastic differentiation of preosteoblasts to a plateau level that could only be achieved by 200 ng/ml of BMP2 [15]. In chapter 2, we also showed that BMP2/7 induced a significantly higher level of cell viability than both the controls; however, at the same concentrations, the two homodimeric BMPs could not. Consequently, it was not unexpected to observe that BMP2/7 could restore cell viability. However, how BMP2/7 and ATRA interact with each other to modulate cell viability (possibly through modulating mitochondrial function) requires further investigation.
BMPs, especially BMP2, 4, 6, 7, and 9 can induce **de novo** bone formation, even in ectopic sites. BMP2, together with an absorbable collagen sponge (ACS) has been approved for the clinical promotion of bone regeneration [16]. Therefore, it is not inconceivable to adopt BMPs to antagonize the inhibitive effect of ATRA and promote osteoblastogenesis. The signaling pathways responsible for BMP-induced osteoblastogenesis are different from those for natural skeletal development, where RUNX2 is an upstream regulator of Osterix and DLX5 expression. In contrast, during BMP-induced osteoblastogenesis, BMP-triggered p-SMAD1/5 could directly upregulate DLX5, which is an upstream regulator and could independently regulate both RUNX2 and Osterix [17]. In chapter 2, for the first time, we showed that heterodimeric BMP2/7 induced significantly higher expression of **DLX5** than homodimeric BMP2 or BMP7. Consistently, significantly higher SMAD1/5 gene expression, as a positive feedback, was also induced by BMP2/7 compared with the homodimeric BMPs at a later time point. The subsequent **RUNX2** expression induced by BMP2/7 was significantly higher than that induced by BMP7 on day 4, and also higher than that induced by both BMP2 and BMP7 on day 7. BMP2/7-induced Osterix was higher than that induced by BMP2 and significantly higher than that induced by BMP7. These data clearly indicated that BMP2/7 could induce higher or significantly higher levels of genes encoding proteins from canonical signaling pathways and higher positive feedback signaling than BMP2 and BMP7 at the same concentration. Our data also showed that BMP2/7 could trigger significantly higher levels of p-SMAD1/5 than BMP2 (data not shown), which indirectly favors the hypothesis that the higher potency of BMP2/7 might be attributed to its significantly higher binding affinity to both type I and type II receptors compared with the homodimers [18]. Such enhanced signaling led to significantly higher osteoblastogenesis, as assessed by collagen expression, ALP, OCN, and the area of mineralized nodule. However, unexpectedly, BMP2/7 was not superior to the homodimers in antagonizing the inhibitory effect of ATRA. Our data showed that BMP2/7 was only advantageous in elevating the cell viability of preosteoblasts compared with BMP2 or BMP7 at certain time points. In comparison with ATRA alone, the addition of all the three BMPs significantly rescued the late differentiation marker (OCN) and the final differentiation marker (mineralized nodules). However, ATRA could significantly suppress OCN and mineralization, irrespective of the potency of the different BMPs. Interestingly, the extent of ATRA’s suppressive effect seemed not to be associated with BMP potency. On day 28, for example, the addition of ATRA suppressed the area of mineralized nodules from 71.2 ± 6.1% in the BMP2/7 group to 7.5 ± 0.6%, and from 18.0 ± 2.2% in BMP2 group to 8.0 ± 0.2%. It seemed that ATRA could generalize the effect of BMP, resulting in the transition of the OCN level and the area of mineralized nodules to a level similar to that of the control group (No ATRA, No BMP). Contrastingly, ATRA either enhanced or did not influence the ALP levels induced by BMP.

Until now, the mechanism by which BMP and ATRA interact with each other in the process of osteoblastogenesis has remained largely unknown. Our data suggested that the transcription of **DLX5** gene, the direct target of p-SMAD1/5, was significantly compromised by ATRA. One study in embryo cells suggested one potential mechanism for such an effect of ATRA: The signal duration of p-SMAD51/5 was significantly affected by ATRA via promotion of GADD45 and MAPK-mediated ubiquitination and proteasomal degradation of p-SMAD1/5 [19]. However, our data did not verify that the duration of p-SMAD1/5 was significantly compromised by ATRA in MC3T3-E1 preosteoblasts (data not shown). Instead, Hisada et al. showed that ATRA could enhance SMAD-binding elements (SBE) activity, although RA itself had no effect on SBE-luciferase activity in C3H10T1/2 Cells [8]. Conversely, SMAD1/4 could also promote the transcriptional
activity of RARE. In these studies, MSX2 was shown to be responsible for the synergistic effect of ATRA and BMP in promoting ALP activity. Such an effect of MSX2 might depend on its intrinsic DNA binding activity [20]. Furthermore, MSX2 suppressed adipogenic differentiation of MSCs via mechanisms that were independent of the intrinsic MSX2 DNA-binding activity, in which MSX2 participated in protein-protein interactions with C/EBPα, which inhibited transcriptional activation of PPAR [20]. By contrast, MSX2 was also shown to antagonize the transcriptional activity of DLX5 in promoting the expression of RUNX2 [17] and OCN [21]. In addition, MSX2 could occupy the promoter of OCN, which led to suppression of its transcription [22]. In conclusion, although BMP2/7 was more potent in promoting osteoblastogenesis, BMP2/7 was not superior to homodimeric BMPs in antagonizing the inhibitive effects of ATRA on osteoblastogenesis. ATRA might affect BMP-induced osteoblastogenesis through a series of molecular mechanisms, including suppression of DLX5 transcription.

2. The combined effects of BMPs and ATRA on osteoclastogenesis

Bone regeneration comprises a delicate balance between bone formation and bone resorption, in which osteoblasts and osteoclasts play essential roles to couple these two processes functionally and quantitatively [23]. Overstimulated osteoclast activity may result in imbalanced bone turnover [24]. High doses of BMP2 or BMP7 need to be administrated systemically to treat bone loss or osteoporosis; however, this poses a risk of systemic adverse effects. Recently, we reported that low concentrations of BMP2/7 had a higher potency on osteoblastogenesis compared with BMP2 or BMP7 homodimers alone [15]. However, we found that low-dose BMP2/7 enhanced osteoclast formation and function, which might accelerate bone loss instead of preventing it [25]. ATRA has been reported to inhibit osteoclast formation and activity [26-28]. Therefore, the combination of ATRA and low dose of BMP2/7 might be more effective to treat hyperactive osteoclast-induced bone loss. In chapter 3, we tested the effect of the combination of ATRA and BMP2/7 on osteoclast formation and activity in vitro. We found that BMP2/7 enhanced osteoclast formation and activity, and ATRA was able to block osteoclast formation and activity in the presence of BMP2/7. We found that ATRA-mediated inhibition of osteoclastogenesis did not act not via RARα, RARβ, or RARγ-related signaling. This inhibition might act directly via RANK-RANKL or NFATc1-related signaling. This is the first study to show the effect of a combination of ATRA and BMP2/7 on osteoclast formation and activity. Our findings suggested the possibility of using a combination of ATRA and BMP2/7 to treat hyperactive osteoclast-mediated bone loss. We found that ATRA could antagonize the stimulatory effect of heterodimeric BMP2/7 on the expression of osteoclastogenic genes CALC, ACP5, and CTSK. Collectively, our data suggested that ATRA is a key regulator of BMPs-induced osteoclastogenesis. Therefore, these data revealed that ATRA could be combined with BMPs to treat bone tumors or osteophytes.

3. Traditional Chinese Medicine: NGR1 for bone regeneration

The effective doses of homodimeric BMPs to induce bone formation are very high (e.g. up to several milligrams) [5,6], which leads to a substantial economic burden and potential side effects, such as overstimulation of osteoclastic activity in surrounding tissue and ectopic bone formation in unintended areas [7,8]. By comparison, Traditional Chinese Medicine is an attractive and promising alternative, possessing high bioactivity and low side effects. In modern Traditional Chinese Medicine, effective compositions are extracted from certain herbs to treat bone diseases. PNS has shown a wide range of pharmacological actions, such as angiogenic, antineoplastic, neuroprotective, anti-inflammatory and immune-modulatory effects [36-40]. NGR1,
a unique and abundant component of PNS [41], has already been used to inhibit hypoxia-hypercapnia-induced vasoconstriction and to protect cells against intestinal ischemia and reperfusion [42, 43]. NGR1’s clinical applications include treatment of osteoporosis and vascular disorders [44]. However, this is the first time that the function of NGR1 on osteoblastogenesis has been investigated. The effect of NGR1 showed a dose-dependent, bell-shaped pattern when modulating cell proliferation and ALP activities. The optimal concentration was determined as 50 μg/ml. Interestingly, the effect of NGR1 on OCN expression showed a dose-dependent increase. A time-dependent and dose-dependent pattern was also observed for OCN gene expression.

After culturing pre-osteoblasts in mineralization medium for 21 days, we observed that 1000 μg/ml of NGR1 produced more bone nodules than other concentrations. A time-dependent and dose-dependent pattern was also observed for mineralization. NGR1 at 1000 μg/ml significantly enhanced mineralization by 5.9 fold, whereas ALP was inhibited. In contrast, the effect of NGR1 in promoting mineralization was consistent its promotion of OCN expression. This result suggested that the promoting effect of NGR1 on mineralization could be partially attributed to its promoting effect on OCN, but not ALP activity. These results suggested that high levels of NGR1 could significantly enhance osteoblastogenesis, thereby having a promising application potential to aid bone regeneration and implant osteointegration for patients. Hitherto, the molecular mechanisms accounting for the promoting effects of NGR1 on osteoblastogenesis remained unknown. RUNX2, a key modulator of osteogenic differentiation, controls osteoblast proliferation and promotes the transition from a proliferative to a post-proliferative stage before osteoblast differentiation [45, 46]. The expression of RUNX2 significantly increased under stimulation by 50 μg/ml NGR1, whereas it significantly decreased in the presence of 1000 μg/ml NGR1. This result suggested that the highest mineralization in response to 1000 μg/ml NGR1 was not due to the upregulation of RUNX2. A previous report showed that the induction of ALP activity was mediated through the activation of the p38 MAPK pathway, a SMAD-independent signaling pathway [47]. OCN is a late differentiation marker for osteoblastogenesis, which is modulated by osterix [48]. It is plausible that NGR1 at different concentrations could modulate p38 MAPK and osterix differently. Therefore, NGR1 might be suitable to repair bone defects.

**Osteoinductive agents with antibacterial properties**

1. The antibacterial efficacy of osteoinductive the BMP2-BioCaP/HACC complex

   With the development of the economy and transportation, road traffic-related high-energy injuries account for nearly 1.2 million deaths worldwide. High-energy injuries can easily develop into severe infected bone defects when a bacterial infection is introduced because of compromised immune protection. The treatment of infected critical-sized bone defects remains a significant challenge in orthopedic, oral, and maxillofacial surgery. To overcome it and consequently repair the infected critical-sized bone defect, we believe the ideal local biomaterial system should meet the following requirements: 1) The biomaterial system can function as both an antibiotic carrier and a bone substitute, to not only clear the infection, but to also contribute to the subsequent bone regeneration process; 2) the antibiotics used for local delivery should have a broad spectrum of activity and a low rate of bacteria resistance; 3) the antibiotic should also be delivered at its optimal concentration, at which it reaches a balance between cellular toxicity and antibacterial activity; 4) osteoinductive bone grafts are more favorable to improve bone regeneration; and 5) the release kinetics of antibiotics and osteoinductive agents should meet their different optimal delivery modes.

   Based on this theory, we used our developed osteoinductive bone substitute, BMP2-BioCaP granules,
as an antibiotic carrier to develop an antibacterial and osteoinductive biomaterial: The BMP2-BioCaP/HACC complex. HACC, a strong antibacterial biomaterial, was absorbed on the surface of BMP2-BioCaP; therefore, once the BMP2-BioCaP/HACC complex was implanted in vivo, it could rapidly dissolve into the body fluid to achieve burst release and a transiently high concentration to kill bacteria effectively. Data emerging from in vitro and in vivo studies suggested that inappropriately low antibiotic dosing may contribute to the increasing prevalence of antibiotic resistance [49]. We speculated that long-term, low-dosage of antibiotics offered sufficient time for resistant strains to be selected and significantly proliferated. Therefore, to avoid resistance, a strong antibiotic should be burst released to kill bacteria rapidly, thus minimizing the chance for selected bacteria to develop resistance. Moreover, the ability of HACC to inhibit biofilm formation might also contribute to the prevention of resistance. The burst release of HACC was designed to rapidly kill residual bacteria in infected bone defects without inducing bacterial resistance. To function as an effective osteoinductive agent, BMP2 needs to be released slowly and continuously at a low concentration [50]. The bone formation induced by the BMP2-BioCaP/HACC complex also supported the view that it had achieved the optimal delivery mode of BMP2. In the present study, the feasibility of the BMP2-BioCaP/HACC complex applied in bone tissue engineering was assessed. The results suggested that the BMP2-BioCaP/HACC complex is particularly suitable to repair infected bone regeneration because of the rapid killing of residual bacteria and the effective promotion of osteogenesis. In our ongoing studies, in vivo infected-bone-defect models will be used to evaluate the antibacterial efficacy of the BMP2-BioCaP/HACC complex.

2. The effect of the antimicrobial agent: pH-sensitive AgNP

Other adverse conditions are caused by chronic bacterial infections, such as periodontitis and peri-implantitis. Peri-implantitis can lead to the failure of implants, and imposes health and financial burdens on patients and health providers. Standard treatments for infected bone defects include the removal of necrotic bone fragments, local and/or systemic administration of antibiotics [51], and reconstruction of the bone defects by bone grafts [52, 53]. However, these treatments are time-consuming (often taking several months to years) [54] and do not always yield satisfactory outcomes [55, 56]. Compared with the antibiotics that are used clinically, many novel antibacterial biomaterials showed promising application potential because of their broader bactericidal spectrum, lack of resistance, and good biocompatibility. The development of novel biomaterials with both antibacterial and osteoinductive properties is essential to provide a viable treatment option. AgNPs are clusters of silver atoms with diameters ranging from 1 to 100 nm. AgNPs have been developed for medical applications because of their antimicrobial, anti-inflammatory, biocompatible, and wound-healing-favoring properties [57]. Compared with the high toxicity of silver ions, AgNPs have larger surface area-to-volume ratios, greater efficacy against bacteria [58] and, most importantly, a lower toxicity to humans [59]. The powerful antimicrobial activities of AgNPs can be attributed to the following orchestrated mechanisms: (1) damage to bacterial membranes; (2) inhibition of DNA replication, protein synthesis and enzymatic activity; and (3) alteration of cell respiration [60]. Compared with traditional antibiotics, AgNPs have a broader spectrum of antibacterial activity. Furthermore, bacterial resistance to AgNPs is very rare [61], which suggests the presence of multiple bactericidal mechanisms acting synergistically. In chapter 7, we developed a novel a pH-dependent silver nanoparticle releasing titanium implant to control peri-implant infection. Broad-spectrum antimicrobials (AgNPs) were successfully loaded into TNT via pH sensitive AL, without affecting the physicochemical characteristics of TNT. A pH of 5.5, mimicking the pH level in peri-implant surface during bacterial infection,
was able to trigger AgNPs release from TNT. The released AgNPs efficiently controlled bacterial growth *in vitro*. This novel implant was biocompatible and osteoinductive. Implants coated with an antimicrobial agent directly, or via biodegradable polymers, are frequently use to control peri-implant infection [16, 18, 19]. Although TNT provides a larger space for loading antimicrobial agents than Ti, antimicrobials loaded in TNT are exhausted in 30 days [23]. Therefore, drugs directly loaded in TNT cannot be used to control post-implantation infection for longer than one month. Moreover, the antimicrobial agents directly loaded into TNT fail to respond to the subtle variations of the peri-implant microenvironment, such as infection-mediated change in the pH level or the degree of inflammation. Therefore, in this study we loaded AgNPs in TNT via pH-sensitive AL, which allowed drug release based on the peri-implant pH level. The amount of loaded AgNPs was lower than the minimal toxic dose of silver, which indicated the admirable biosafety of this new biomaterial [43]. Our result suggested that infection-dependent decrease in the pH around the TNT-AL-AgNPs could be used as a switch to release the AgNPs.

In conclusion, the strategies discussed in this thesis are useful for bone regeneration in adverse conditions. Based on the discoveries detailed in this thesis, we believe we have built a solid foundation for future clinic applications.
References


